12-14-99 Docket No. 50875-F-PCT-US/JPW/SHS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE A/BOX SEQ

Honorable Assistant Commissioner for Patents
Washington, D.C. 20231

S I R: December 13, 1999

Transmitted herewith for filing are the specification and claims of the patent application of:

Graham P. Allaway, Virginia M. Litwin, Paul J. Maddon and William C. Olson for Inventor(s)

METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS
Title of Invention

Also enclosed are:

X 10 sheet(s) of ___informal X formal drawings.

X Oath or declaration of Applicant(s). (unsigned)

X A power of attorney (unsigned)

___ An assignment of the invention to _____

X A Preliminary Amendment

___ A verified statement to establish small entity status under 37 C.F.R. §1.9 and §1.27.

The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE SMALL ENTITY	RATE OTHER ENTITY		FEE SMALL ENTITY	FEE OTHER ENTITY
Total Claims	4 -20	=	0	X	$ 9.00	$18.00	=	$ 0	$ 0
Independent Claims	1 -3	=	0	X	$39.00	$78.00	=	$ 0	$ 0
Multiple Dependent Claims Presented:	___Yes X No				$130.00	$260.00	=	$ 0	$ 0
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Applicants: Graham P. Allaway, Virginia M. Litwin, Paul J. Maddon and William C. Olson
Serial No.: Not Yet Known
Filed: December 13, 1999

Letter of Transmittal
Page 2

X A check in the amount of $ 380.00 to cover the filing fee.

___ Please charge Deposit Account No. _____ in the amount of $_____.

X The Commissioner is hereby authorized to charge any additional fees which may be required in connection with the following or credit any over-payment to Account No. 03-3125:

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X Patent application processing fees under 37 C.F.R. §1.17.

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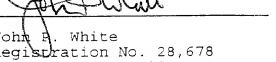
X Three copies of this sheet are enclosed.

___ A certified copy of previously filed foreign application No. _____ _____ filed in _____ on _____. Applicant(s) hereby claim priority based upon this aforementioned foreign application under 35 U.S.C. §119.

X Other (identify) Express Mail Certificate of Mailing bearing label No. EL278887487US; One loose set of figures; computer diskette containing CRF; Statement in Accordance with 37 CFR 1.821 (f)

Respectfully submitted,



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Dkt. 50875-F-PCT-US/JPW/SHS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Graham P. Allaway, et al.

Serial No.: Not Yet Known (Continuation application of PCT/US98/12331, filed June 12, 1998)

Filed : December 13, 1999

For : METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS

1185 Avenue of the Americas
New York, New York 10036
December 13, 1999

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

EXPRESS MAIL CERTIFICATE OF MAILING FOR ABOVE-IDENTIFIED APPLICATION

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SIR:

PRELIMINARY AMENDMENT

Please amend the subject application as follows:

In the specification:

On page 1, line 1, after the words "This application is a" and before the words "continuation-in-part" please insert the following:

--continuation application of PCT International Application No. PCT/US98/12331, filed June 12, 1998, which is a--.

In the claims:

Please cancel claims 1-60 without prejudice to applicants' right to pursue the subject matter of these claims in a later-filed application. Please add new claims 61-64 as follows.

--61. (New) A method of inhibiting HIV-1 infection of CD4+ cells which comprises contacting CD4+ cells with a non-chemokine peptidyl agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 or an HIV-1 infected cell to the CD4+ cells is inhibited, so as to thereby inhibit

HIV-1 infection of the CD4+ cells, provided that the nonchemokine peptidyl agent is not a bicyclam or a derivative thereof.--

--62. (New) The method of claim 61, wherein the non-chemokine peptidyl agent is an oligopeptide.--

--63. (New) The method of claim 61, wherein the non-chemokine peptidyl agent is a polypeptide.--

--64. (New) The method of claim 63, wherein the non-chemokine peptidyl agent is an antibody or portion of an antibody.--

Remarks:

Claims 1-60 were pending in the subject application. Applicants have hereinabove canceled claims 1-60 without prejudice to their right to pursue the subject matter of these claims in a later-filed application and added new claims 61-64 as follows. Support for these amendments may be found inter alia in the specification as follows: claim 61: page 13, lines 1-6, page 14, lines 6-10 and 21-22; claim 62: page 14, lines 13-14; claim 63, lines 14-15; claim 64, lines 15-17. Accordingly, claims 61-64 involve no issue of new matter and entry of this amendment is respectfully requested.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, in addition to the enclosed filing fee of $380.00, is deemed necessary in connection with the filing of this Preliminary Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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Application for United States Letters Patent

To all whom it may concern:

Be it known that (we) Graham P. Allaway, Virginia M. Litwin, Paul J. Maddon and William C. Olson

have invented certain new and useful improvements in

METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS

of which the following is a full, clear and exact description.

METHOD FOR PREVENTING HIV-1 INFECTION OF CD4$^+$ CELLS

This application is a continuation-in-part application of U.S. Serial No. 08/876,078, filed June 13, 1997, the contents of which is hereby incorporated by reference.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each series of experiments.

Background of the Invention

Chemokines are a family of related soluble proteins of molecular weight between 8 and 10KDa, secreted by lymphocytes and other cells, which bind receptors on target cell surfaces resulting in the activation and mobilization of leukocytes, for example in the inflammatory process. Recently, Cocchi et al. demonstrated that the chemokines RANTES, MIP-1α and MIP-1β are factors produced by CD8$^+$ T lymphocytes which inhibit infection by macrophage-tropic primary isolates of HIV-1, but not infection by laboratory-adapted strains of the virus (1). These chemokines are members of the C-C group of chemokines, so named because they have adjacent cysteine residues, unlike the C-X-C group which has a single amino acid separating these residues (2). While Cocchi et al. found that expression of HIV-1 RNA was suppressed by treatment with the chemokines, they did not identify the site of action of these molecules.

A resonance energy transfer (RET) assay of HIV-1 envelope glycoprotein-mediated membrane fusion was used to determine whether fusion mediated by the envelope glycoprotein from the primary macrophage-tropic isolate of HIV-1_{JR-FL} would be specifically inhibited by chemokines, when compared with fusion mediated by the envelope glycoprotein from the

laboratory-adapted T lymphotropic strain HIV-1_{LAI}. As described below, it was demonstrated that this is indeed the case. This demonstrates that some chemokine receptors are fusion accessory molecules required for HIV-1 infection. Previous studies have indicated that unidentified cell surface molecules are required for virus entry in addition to the HIV-1 receptor, CD4. While CD4 is required for HIV-1 attachment, the accessory molecules are required for the membrane fusion step of entry. These accessory molecules are generally expressed only on human cells, so HIV-1 does not infect non-human $CD4^+$ cells (3-6). Moreover it is possible to complement non-human $CD4^+$ cells by fusing them (using polyethylene glycol) with $CD4^-$ human cells, resulting in a heterokaryon which is a competent target for HIV-1 envelope-mediated membrane fusion (7,8). These studies have been performed using laboratory-adapted T lymphotropic strains of the virus.

In some cases, it appears that fusion accessory molecules are found on a subset of human $CD4^+$ cells and are required for infection by HIV-1 isolates with particular tropisms. For example, macrophage-tropic primary strains of HIV-1 such as HIV-1_{JR-FL} may have different requirements for accessory molecules compared with laboratory-adapted T lymphotropic strains such as HIV-1_{LAI}. This phenomenon may explain differences in tropism between HIV-1 strains.

The current invention comprises a series of new therapeutics for HIV-1 infection. It was demonstrated for the first time that chemokines act at the fusion step of HIV-1 entry and specifically inhibit membrane fusion mediated by the envelope glycoprotein of primary macrophage-tropic primary viral isolates, not laboratory-adapted T lymphotrophic strains of the virus. Primary macrophage-tropic isolates of the virus are of particular importance since they are the strains usually involved in virus transmission, and may have particular importance in the pathogenesis of HIV-1 infection.

These results were obtained using a resonance energy transfer (RET) assay of HIV-1 envelope-mediated membrane fusion. Moreover, this assay is used to identify non-chemokines, including fragments of chemokines and modified chemokines, that inhibit HIV-1 envelope glycoprotein-mediated membrane fusion and thereby neutralize the virus, yet do not induce an inflammatory response.

Summary of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to $CD4^+$ cells which comprises contacting CD4 cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of $CD4^+$ cells which comprises contacting $CD4^+$ cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited, thereby inhibiting the HIV-1 infection.

This invention further provides non-chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to $CD4^-$ cells.

This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide. In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonypeptidyl agent.

In addition, this invention provides pharmaceutical compositions comprising an amount of the above non-chemokine agents or agents capable of binding to fusin effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the $CD4^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the

other receptor.

This invention also provides a pharmaceutical composition comprising an amount of the above-described composition of matter effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing the likelihood of HIV-1 infection in a subject comprising administering an above-described pharmaceutical composition to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering an above-described pharmaceutical composition to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a $CD4^+$ cell which comprise: (a) contacting (i) a $CD4^+$ cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the $CD4^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the

product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to $CD4^+$ cells.

Brief Description of the Figures

Figure 1. **Membrane fusion mediated by the HIV-1_{JR-FL} envelope glycoprotein is inhibited by RANTES, MIP-1α and MIP-1β.**

%RET resulting from the fusion of PM1 cells and HeLa-env_{JR-FL} (■) or HeLa-env_{LAI} (◆) was measured in the presence and absence of recombinant human chemokines at a range of concentrations: RANTES (80 - 2.5 ng/ml), MIP-1α (400 - 12.5 ng/ml) and MIP-1β (200 - 6.25 ng/ml), as indicated. Chemokines were added simultaneously with the cells at the initiation of a four hour incubation. Data are representative of more than three independent experiments which were run in duplicate. The percent inhibition of RET is defined as follows:

% Inhibition = 100 . [(Max RET - Min RET) - (Exp RET- Min RET)]/(Max RET - Min RET)

where Max RET is the %RET value obtained at four hours with HeLa-env cells and CD4-expressing cells in the absence of an inhibitory compound; Exp RET is the %RET value obtained for the same cell combination in the presence of an inhibitory compound and Min RET is the background %RET value obtained using HeLa cells in place of HeLa envelope-expressing cells.

Figure 2. **CD4:HIV-1 gp120 binding in the presence of human chemokines.**

The binding of soluble human CD4 to HIV-1_{LAI} and HIV-1_{JR-FL} gp120 was determined in an ELISA assay in the presence and absence of the monoclonal antibody OKT4A or recombinant human chemokines at

a range of concentrations, identical to those used in the RET inhibition studies of Figure 1: OKT4A (62 - 0.3 nM), RANTES (10.3 - 0.3 nM), MIP-1α (53.3 - 2.9 nM), and MIP-1β (25.6 - 0.8 nM). Inhibitors were added simultaneously with biotinylated HIV-1 gp120 to soluble CD4 coated microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). Following a two hour incubation at room temperature and extensive washing, an incubation with streptavidin-horseradish peroxidase was performed for one hour at room temperature. Following additional washes, substrate was added and the OD at 492 nm determined in an ELISA plate reader. Data are representative of two independent experiments which were run in quadruplicate.

Figure 3. **Specificity. time course and stage of ß-chemokine inhibition of HIV-1 replication.**

(a) PM1 cells (1 x10^6) were preincubated with RANTES + MIP- 1α + MIP-1ß (R/Mα/Mß; 100ng/ml of each) for 24h (-24h) or 2h (-2h), then washed twice with phosphate buffered saline (PBS). HIV-1 (BaL env-complemented) virus (50ng of p24; see legend to Table 1) was added for 2h, then the cells were washed and incubated for 48h before measurement of luciferase activity in cell lysates as described previously (10,11). Alternatively, virus and R/Mα/Mß were added simultaneously to cells, and at the indicated time points (1h, 3h, etc) the cells were washed twice in PBS, resuspended in culture medium and incubated for 48h prior to luciferase assay. Time 0 represents the positive control, to which no ß-chemokines were added. +2h represents the mixture of virus with cells for 2h prior to washing twice in PBS, addition of R/Mα/Mß and continuation of the

culture for a further 48h before luciferase assay.

(b) PM1 cells ($1x10^6$) were infected with HIV-1 (500pg p24) grown in CEM cells (NL4/3; lanes 1-4) or macrophages (ADA; lanes 5-8), in the presence of 500ng/ml of RANTES (lanes 1 and 5) or MIP-1ß (lanes 2 and 6), or with no ß-chemokine (lanes 4 and 8). Lanes 3 and 7 are negative controls (no virus). All viral stocks used for the PCR assay were treated with DNAse for 30 min at 37°C, and tested for DNA contamination before use. After 2h, the cells were washed and resuspended in medium containing the same ß-chemokines for a further 8h. DNA was then extracted from infected cells using a DNA/RNA isolation kit (US Biochemicals). First round nested PCR was performed with primers: U3+, 5'-CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG-3' (SEQ ID NO:1) preGag, 5'-AGCAAGCCGAGTCCTGCGTCGAGAG-3' (SEQ ID NO:2) and the second round with primers: LTR-test, 5'-GGGACTTTCCGCTGGGGACTTTC 3'(SEQ ID NO :3) LRC2, 5'-CCTGTTCGGGCGCCACTGCTAGAGATTTTCCAC 3' (SEQ ID NO:4) in a Perkin Elmer 2400 cycler with the following amplification cycles: 94°C for 5 min, 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s, 72°C for 7 min. M indicates 1kb DNA ladder; 1, 10, 100, 1000 indicate number of reference plasmid (pAD8) copies. The assay can detect 100 copies of reverse transcripts.

Figure 4: **HIV-1 env-mediated membrane fusion of cells transiently expressing C-C CKR-5.**

Membrane fusion mediated by ß-chemokine receptors expressed in HeLa cells was demonstrated as follows: Cells were transfected with control plasmid pcDNA3.1 or plasmid pcDNA3.1-CKR constructs using lipofectin (Gibco BRL). The

pcDNA3.1 plasmid carries a T7-polymerase promoter and transient expression of ß-chemokine receptors was boosted by infecting cells with $1x10^7$ pfu of vaccinia encoding the T7-polymerase (vFT7.3) 4h post-lipofection (9). Cells were then cultured overnight in R18-containing media and were tested for their ability to fuse with HeLa-JR-FL cells (filled columns) or HeLa-BRU cells (hatched column) in the RET assay. The %RET with control HeLa cells was between 3% and 4% irrespective of the transfected plasmid.

Figure 5 **Membrane fusion mediated by the HIV_{LAI} envelope glycoprotein is inhibited by SDF-1.**

% RET resulting from the fusion of PM1 cells and HeLa-env_{JR-FL} or HeLa-env_{LAI} cells (as indicated on the graph) was measured in the presence of recombinant SDF-1α(Gryphon Science, San Francisco) at the indicated concentrations. Experimental method as described in the legend to Fig. 1.

Figure 6. **Flow cytometric analysis of the binding of sCD4-gp120 complexes to (a)CCR5$^-$ and (b)CCR5 L1.2 cells, a murine pre-B lymphoma line.**

Cells are incubated for 15 min. with equimolar (~100nM) mixtures of sCD4 and biotinylated $HIV\text{-}1_{JR\text{-}FL}$ gp120 and then stained with a streptavidin-phycoerythrin conjugate, fixed with 2% paraformaldehyde, and analyzed by FACS. Cell number is plotted on the y-axis.

Figure 7. **Inhibition of HIV-1 envelope-mediated cell fusion by the bicyclam JM3100, measured using the RET assay, with the cell combinations indicated.**

Figure 8. **Binding of CD4 and gp120 to CCR5.**

Recombinant soluble CD4 (sCD4) and recombinant gp120 were added in a range of concentrations either individually or as an equimolar molecular complex to recombinant L1.2 cells that express human CCR5 on their cell surface. The recombinant proteins were biotinylated as indicated. Binding was detected by adding a streptavidin-phycoerythrin conjugate and measuring the fluoroescence emission at 590 nm following excitation at 530 nm. The following species were tested:

b-LAI:sCD4: complex formed between scD4 and biotinylated HIV-1_{LAI} gp120

b-JR-FL:sCD4: complex formed between sCD4 and biotinylated HIV-1_{JR-FL} gp120

b-sCD4 alone: biotinylated sCD4 added in the absence of gp120

b-JR-FL alone: biotinylated HIV-1_{JR-FL} gp120 added inthe absence of sCD4

These data demonstrate that complexation of soluble CD4 and gp120 is necessary for CCR5 binding, as minimal binding is observed for sCD4 or gp120 alone. The data further demonstrate that binding is observed for sCD4-gp120 complexes when the gp120 is derived from macrophage-tropic (e.g., JR-FL) but not T cell-tropic (e.g., LAI) strains of HIV, as expected from the known relationship between HIV-1 tropism and co-receptor usage. All data have been corrected for residual background binding to nontransfected CCR5- L1.2 cells. To enhance chemokine receptor expression, both transfected and parental L1.2 cells were treated with sodium butyrate prior to assay (Wu et al., J. Exp. Med. 185:1681)

Figure 9. **The CCR5 Binding assay identifies and determines the potency of inhibitors of the gp120-CCR5 interaction.**

HIV-1 Inhibitory monoclonal antibodies were added in a range of concentrations to reecmbinant L1.2 cells that express human CCR5 on their cell surface and used to compete the binding of a complex formed between sCD4 and biotinylated HIV-1_{JR-FL} gp 120, whose binding was detected using a streptavidin-phycoerythrin conjugate. PA-8, -9, -10, -11 and -12 are Progenics' monoclonal antibodies that inhibit HIV-1 entry, while 2D7 is a commercially available (Pharmingen, San Diego, CA) ant-CCR5 monoclonal antibody that inhibits HIV-1 entry. To enhance chemokine receptor expression, both transfected and parental L1.2 cells were treated with sodium butyrate prior to assay (Wu et al., J. Exp. Med. 185:1681).

Detailed Description of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to CD4$^+$ cells which comprises contacting CD4 cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4$^+$ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of CD4$^+$ cells which comprises contacting CD4$^+$ cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4$^+$ cells is inhibited, thereby inhibiting the HIV-1 infection.

In this invention, a chemokine means RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. A chemokine receptor means a receptor capable of binding RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. Such chemokine receptor includes but not limited to CCR5, CXCR4, CCR3 and CCR-2b.

Throughout this application, the receptor "fusin" is also named CXCR4 and the chemokine receptor C-C CKR5 is also named CCR5.

The HIV-1 used in this application unless specified will mean clinical or primary or field isolates or HIV-1 viruses which maintain their clinical characteristics. The HIV-1 clinical isolates may be passaged in primary peripheral blood mononuclear cells. The HIV-1 clinical isolates may be macrophage-trophic.

The non-chemokine agents of this invention are capable of binding to chemokine receptors and inhibiting fusion of HIV-1 to CD4$^+$ cells. The non-chemokine agents include, but are not limited to, chemokine fragments and chemokine derivatives and analogues, but do not include naturally occurring chemokines. The non-chemokine agents include

multimeric forms of the chemokine fragments and chemokine derivatives and analogues or fusion molecules which contain chemokine fragments, derivatives and analogues linked to other molecules.

The non-chemokine agents do not include bicyclams and their derivatives as described in U.S.Patent No. 5,021,409, issued June 4, 1991, the content of which is incorporated by reference into this application. Some bicyclam derivatives have been previously described with antiviral activities (15, 16).

In an embodiment of this invention, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the non-chemokine agent is an antibody or a portion thereof. Antibodies against the chemokine receptor may easily be generated by routine experiments. It is also within the level of ordinary skill to synthesize fragments of the antibody capable of binding to the chemokine receptor. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

Non-chemokine agents which are purely peptidyl in composition can be either chemically synthesized by solid-phase methods (Merrifield, 1966) or produced using recombinant technology in either prokaryotic or eukaryotic systems. The synthetic and recombinant methods are well known in the art.

Non-chemokine agents which contain biotin or other nonpeptidyl groups can be prepared by chemical modification of synthetic or recombinant chemokines or non-chemokine agents. One chemical modification method involves periodate oxidation of the 2-amino alcohol present on chemokines or non-chemokine agents possessing serine or threonine as their N-terminal amino acid (Geophegan and Stroh, 1992). The resulting aldehyde group can be used to link peptidyl or

non-peptidyl groups to the oxidized chemokine or non-chemokine agent by reductive amination, hydrazine, or other chemistries well known to those skilled in the art.

As used herein, a N-terminus of a protein should mean the terminus of the protein after it has been processed. In case of a secretory protein which contains a cleavable signal sequence, the N-terminus of a secretory protein should be the terminus after the cleavage of a signal peptide.

This invention provides a method of identifying these non-chemokine agents. One way of identifying such agents, including non-peptidyl agents, that bind to a chemokine receptor and inhibit fusion of HIV-1 to $CD4^+$ cells is to use the following assay: 1) Incubate soluble CD4 with biotinylated gp120 from HIV-1_{JR-FL} or HIV-1_{IIIA} ; 2) Incubate this complex with CCR5 or CXCR4-expressing cells (for HIV-1_{JR-FL} or HIV-1_{IIIA} gp120s, respectively) that do not express CD4, in the presence of absence of a candidate inhibitor; 3) Wash and then incubate with streptavidin-phycoerythrin; and 4) Wash and then measure the amount of bound gp120 using a flow cytometer or fluorometer and calculate the degree of inhibition of binding by the inhibitor.

Alternative methods to detect bound gp120 can also be used in place of the biotinylated gp120-streptavidin-phycoerythrin method described above. For example, peroxidase-conjugated gp120 could be used in place of the biotinylated gp120 and binding detected using an appropriate colorimetric substrate for peroxidase, with a spectrometric readout.

This invention further provides the non-chemokine agents identified by the above methods.

This invention provides a non-chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of

HIV-1 to CD4$^+$ cells with the proviso that the agent is not a known bicyclam or its known derivatives. In an embodiment, the non-chemokine is a polypeptide. In a further embodiment, this polypeptide is a fragment of the chemokine RANTES (Gong et al., 1996). In a still further embodiment, the polypeptide may also comprise the RANTES sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first eight N-terminal amino acids of the RANTES sequence (SEQ ID NO:5).

In a separate embodiment, the polypeptide may comprise the MIP-1ß sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first seven, eight, nine or ten N-terminal amino acids of the MIP-1ß sequence.

In another embodiment of non-chemokine agent, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide. In a separate embodiment, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by removing the N-terminal alanine and replaced it by serine or threonine and additional amino acid or oligopeptide or nonpeptidyl moiety. In a further embodiment, the additional amino acid is methionine.

As described infra in the section of Experimental Details, a cofactor for HIV-1 fusion and entry was identified and designated "fusin" (Feng et al., 1996). This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide.

In a further embodiment, the polypeptide comprises SDF-1 with deletion of the N-terminal amino acids of said sequence. The deletion may be the first six, seven, eight, or nine N-terminal amino acids of the SDF-1 sequence.

This invention also provides the above non-chemokine agent, wherein the polypeptide comprises SDF-1 sequence with the N-terminal sequence modified to produce antagonistic effect to SDF-1. One modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with biotin. Another modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with methionine. A further modification is to add the N-terminus of SDF-1 with a methionine before the terminal glycine.

In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonpeptidyl agent.

The agents capable of binding to fusin may be identified by screening different compounds for their capability to bind to fusin in vitro.

A suitable method has been described by Fowlkes, et al. (1994), international application number: PCT/US94/03143, international publication number: WO 94/23025, the content of which is incorporated by reference into this application. Briefly, yeast cells having a pheromone system are engineered to express a heterologous surrogate of a yeast pheromone system protein. The surrogate incorporates fusin and under some conditions performs in the pheromone system of the yeast cell a function naturally performed by the corresponding yeast pheromone system protein. Such yeast cells are also engineered to express a library of peptides whereby a yeast cell containing a peptide which binds fusin exhibits modulation of the interaction of surrogate yeast pheromone system protein with the yeast pheromone system and this modulation is a selectable or screenable event. Similar approaches may be used to identify agents capable of binding to both fusin and the chemokine receptor C-C CKR-5.

This invention also provides pharmaceutical compositions comprising an amount of such non-chemokine agents or agents

capable of binding to fusin effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the $CD4^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor. In an embodiment, the cell surface receptor is CD4. In another embodiment, the ligand is an antibody or a portion of an antibody.

This invention also provides a pharmaceutical composition comprising an amount of an above-described composition of matter effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a composition of matter capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol.

This invention also provides a pharmaceutical composition comprising an amount of a composition of matter comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provide methods for reducing likelihood of HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject. This invention also provides methods for treating HIV-1 infection in a subject comprising administering the above-described pharmaceutical compositions to the subject.

This invention also provides methods for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a $CD4^+$ cell which comprise: (a) contacting (i) a $CD4^+$ cell which is labeled with a first dye and (ii) a cell expressing the HIV-1 envelope glycoprotein on its surface which is labeled with a second dye, in the presence of an excess of the agent under conditions permitting the fusion of the $CD4^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of the agent, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is a reduction of resonance energy transfer, when compared with the resonance energy transfer in the absence of the agent, a decrease in transfer indicating that the agent is capable of inhibiting fusion of

HIV-1 to CD4$^+$ cells.

HIV-1 only fuses with appropriate CD4$^+$ cells. For example, laboratory-adapted T lymphotropic HIV-1 strains fuse with most CD4$^+$ human cells. Clinical HIV-1 isolates do not fuse with most transformed CD4$^+$ human cell lines but do fuse with human primary CD4$^+$ cells such as CD4 T lymphocytes and macrophages. Routine experiments may be easily performed to determine whether the CD4$^+$ cell is appropriate for the above fusion assay.

As described in this invention, HIV-1 membrane fusion is monitored by a resonance energy transfer assay. The assay was described in the International Application Number, PCT/US94/14561, filed December 16, 1994 with International Publication Number WO 95/16789. This assay is further elaborated in a United States co-pending application no. 08/475,515, filed June 7, 1995. The contents of these applications are hereby incorporated by reference into this application.

In an embodiment of the above method, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the agent is an antibody or a portion thereof. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

In a separate embodiment, the CD4$^+$ cell is a PM1 cell. In another embodiment, the cell expressing the HIV-1 envelope glycoprotein is a HeLa cell expressing HIV-1_{JR-FL} gp120/gp41.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) contacting an appropriate concentration of an agent with a chemokine receptor or a portion thereof under conditions permitting the binding of the agent to the chemokine receptor; (b) contacting the chemokine receptor

resulting from step (a) with a gp120/CD4 complex under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (c) measuring the amount of bound gp120/CD4 complex wherein a decrease in the amount compared with the amount determined in the absence of the agent indicates that the agent is capable of inhibiting HIV-1 infection.

As used herein, the portion of the chemokine receptor used in the above method is the portion which maintains the capability of binding to HIV, i.e. capable of interaction with the gp120/CD4 complex. It is the intention of this invention to cover hybrid molecules or genetically engineered molecules which comprise this portion or domain of the chemokine receptor.

The gp120/CD4 complex used in the assay may include a truncated form of either molecules or hybrid proteins of molecules as long as the domain for binding to the chemokine receptor is retained.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix; (b) contacting the agent with the fixed chemokine receptor under conditions permitting the binding of the agent to the chemokine receptor; (c) removing the unbound agent; (d) contacting the fixed chemokine receptor resulting in step (c) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound gp120/CD4 complex; and (f) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining

whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix; (b) contacting the agent with the fixed chemokine receptor; (c) contacting the mixture in step (b) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound gp120/CD4 complex; and (e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) contacting the agent with a gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex; (b) contacting the gp120/CD4 complex resulting from step (a) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (c) measuring the amount of bound chemokine receptor, wherein a decrease of the amount when compared with the amount determined in the absence of the agent indicates that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 complex on a solid matrix; (b) contacting the agent with the fixed gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex; (c) removing unbound agent;(d) contacting the fixed gp120/CD4 complex resulting from step (c) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound chemokine receptor; and (f)

comparing the amount determined in step (e) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 on a solid matrix; (b) contacting the agent with the fixed gp120/CD4 complex; (c) contacting the mixture in step (b) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound chemokine receptor; (e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

As used in these assays, CD4 include soluble CD4, fragments of CD4 or polypeptides incorporating the gp120 binding site of CD4 capable of binding gp120 and enabling the binding of gp120 to the appropriate chemokine receptor.

As used in these assay gp120 is the gp120 from an appropriate strain of HIV-1. For example, gp120 from the macrophage tropic clinical isolate HIV-1_{JR-FL} will bind to the chemokine receptor CCR5, whereas gp120 from the laboratory adapted T-tropic strain HIV-1_{LAI} will bind to the chemokine receptor CXCR4.

In a preferred embodiment of the above methods, the CD4 is a soluble CD4. The chemokine receptor which may be used in the above assay includes CCR5, CXCR4, CCR3 and CCR-2b.

In an embodiment, the chemokine receptor is expressed on a cell. In another embodiment, the chemokine receptor is embedded in liposomes. In further embodiment, the chemokine receptor is embedded in a membrane derived from cells

expressing the chemokine receptor. In a preferred embodiment, the cell is a L1.2 cell. In a separate embodiment, the chemokine receptor is purified and reconstituted in liposomes. Such chemokine receptor embedded in the lipid bilayer of liposomes retains the gp120 binding activity of the receptor.

The gp120, CD4 or both may be labelled with a detectable marker in the above assays. Markers including radioisotope or enzymes such as horse radish peroxidase may be used in this invention.

In an embodiment, the gp120 or CD4 or the chemokine receptor is labelled with biotin. In a further embodiment, the biotinylated gp120, or CD4 or the chemokine receptor is detected by: (i) incubating with streptavidin-phycoerythrin, (ii) washing the incubated mixture resulting from step (i), and (iii) measuring the amount of bound gp120 using a plate reader, exciting at 530nm, reading emission at 590nm.

This invention also provides an agent determined to be capable of inhibiting HIV-1 infection by the above methods, which is previously unknown.

This invention also provides a pharmaceutical composition comprising the agent determined to be capable of inhibiting HIV-1 infection by the above methods and a pharmaceutically acceptable carrier. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is a polypeptide. In a still another embodiment, the agent is a nonpeptidyl agent.

This invention also provides a molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising the above determined agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol. This invention also provides a

pharmaceutical composition comprising an amount of the above molecule effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for treating HIV-1 infection in a subject comprising administering the above pharmaceutical composition to the subject.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

FIRST SERIES OF EXPERIMENTS

1) Chemokines inhibit fusion mediated by the envelope glycoprotein from a macrophage-tropic primary isolate of HIV-1 but not from a laboratory-adapted T-lymphotrophic strain of the virus

The chemokines RANTES, MIP-1α and MIP-1β were obtained from R & D systems (Minneapolis, MN). They were tested in the RET assay for ability to inhibit fusion between HeLa-env$_{JR-FL}$ cells (expressing gp120/gp41 from the macrophage tropic isolate HIV-1$_{JR-FL}$) and PM1 cells, or for inhibition of fusion between HeLa-env$_{LAI}$ cells (expressing gp120/gp41 from the laboratory-adapted strain HIV-1$_{LAI}$) and various CD4$^+$ T lymphocyte cell lines. As shown in Figure 1, all three chemokines inhibited fusion mediated by the macrophage tropic virus envelope glycoprotein, but not that mediated by the laboratory-adapted strain envelope glycoprotein.

The ability of the chemokines to block the interaction between CD4 and HIV-1 gp120 which occurs at virus attachment was then tested. It was found that the chemokines did not inhibit this interaction (Figure 2), demonstrating that their blockade of HIV-1 envelope glycoprotein-mediated membrane fusion occurs at the membrane fusion event itself, rather than the initial CD4-gp120 interaction which precedes fusion.

2) Non-chemokine peptides and derivatives that inhibit HIV-1 fusion

The non-chemokines include chemokine fragments and chemokine derivatives that are tested in the RET assay to determine which are active in inhibiting HIV-1 membrane fusion. Particular attention is focused on fragments or derivatives that inhibit HIV-1 fusion but do not activate leukocyte responses. These non-chemokines include:

a) N-terminal derivatives of the chemokines. Addition of residues to the N-terminus of chemokines inhibits the function of these proteins without significantly reducing their ability to bind chemokine receptors. For example, Met-RANTES (RANTES with an N-terminal methionine) has been shown to be a powerful antagonist of native RANTES and is unable to induce chemotaxis or calcium mobilization in certain systems. The mechanism of antagonism appears to be competition for receptor binding (9). Similar results were found using other derivatives of the N terminus of RANTES(9) and also by N-terminal modification of other chemokines, such as IL-8 (a member of the C-X-C chemokines) (10). The current invention includes Met-RANTES and other chemokines derivatised by the addition of methionine, or other residues, to the N-terminus so that they inhibit fusion mediated by the envelope glycoprotein of HIV-1_{JR-FL}, and inhibit infection by many isolates of HIV-1, yet do not activate the inflammatory response.

b) Chemokines with N-terminal amino acids deleted: Chemokine antagonists have been generated by deleting amino acids in the N-terminal region. For example, deletion of up to 8 amino acids at the N-terminus of the chemokine MCP-1 (a member of the C-C chemokine group), ablated the bioactivity of the protein while allowing it to retain chemokine receptor binding and the ability to inhibit activity of native MCP-1 (11,12).

The current invention includes N-terminal deletants of RANTES, MIP-1α and MIP-1β, lacking the biological activity of the native proteins, which inhibit HIV-1 fusion and HIV-1 infection.

c) Other peptides: A series of overlapping peptides (e.g. of 20-67 residues) from all regions of RANTES, MIP-1α and MIP-1β are screened by the same approaches to identify peptides which inhibit HIV-1 fusion most potently without activating leukocytes. Activation of leukocyte responses is

measured following routine procedures (9, 10, 11, 12).

3) Cloning the chemokine receptors

Chemokine receptors required for HIV-1 fusion are cloned by the following strategy. First a cDNA library is made in a mammalian expression vector (e.g. pcDNA3.1 from Invitrogen Corp. San Diego, CA) using mRNA prepared from the PM1 cell line or $CD4^+$ T-lymphocytes or macrophages. Degenerate oligonucleotide probes are used to identify members of the cDNA library encoding members of the chemokine receptor family, for example following previously published methods (2). The vectors containing chemokine receptor cDNAs are then individually expressed in one of several mammalian cell lines which express human CD4 but do not fuse with HeLa-env_{JR-FL} cells (e.g. HeLa-CD4, CHO-CD4 or COS-CD4) or HeLa-env_{LAI} cells (e.g. CHO-CD4 or COS-CD4). Following analysis in the RET assay, clones which gain the ability to fuse with HeLa-env_{JR-FL} or HeLa-env_{LAI} are identified and the coding sequences recovered, for example by PCR amplification, following procedures well known to those skilled in the art. DNA sequencing is then performed to determine whether the cDNA recovered encodes a known chemokine receptor. Following expression of the receptor, monoclonal and polyclonal antibodies are prepared and tested for ability to inhibit infection by a panel of HIV-1 isolates.

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SECOND SERIES OF EXPERIMENTS

The replication of primary, non-syncytium-inducing (NSI) HIV-1 isolates in CD4+ T-cells is inhibited by the C-C ß-chemokines MIP-1α, MIP-1ß and RANTES (1,2), but T-cell line-adapted (TCLA) or syncytium-inducing (SI) primary strains are insensitive (2,3). The ß-chemokines are small (8kDa), related proteins active on cells of the lymphoid and monocyte lineage (4-8). Their receptors are members of the 7-membrane-spanning, G-protein-linked superfamily, one of which (the LESTR orphan receptor) has been identified as the second receptor for TCLA HIV-1 strains, and is now designated fusin (9). Fusin is not known to be a ß-chemokine receptor (7-9).

To study how ß-chemokines inhibit HIV-1 replication, a virus entry assay based on single-cycle infection by an env-deficient virus, NL4/3Δenv (which also carries the luciferase reporter gene), complemented by envelope glycoproteins expressed in trans was used (10,11). Various env-complemented viruses were tested in PM1 cells, a variant of HUT-78 that has the unique ability to support replication of primary and TCLA HIV-1 strains, allowing comparison of envelope glycoprotein functions against a common cellular background (2,12). MIP-1α, MIP-1ß and RANTES are most active against HIV-1 in combination (2,3), and strongly inhibited infection of PM1 cells by complemented viruses whose envelopes are derived from the NSI primary strains ADA and BaL (Table 1a).

Table 1: Inhibition of HIV-1 entry in PM1 cells and CD4+ T-cells by ß-chemokines

	% luciferase activity				
	BaL	ADA	NL4/3	HxB2	MuLV
a)					
PM1 cells					
control without virus	2	2	2	5	3
control with virus	100	100	100	100	100
+R/Mα/Mß (50/50/50)	2	3	92	117	100
+RANTES (100)	1	1	nd	nd	nd
+MIP-1α(100)	54	54	nd	nd	nd
+MIP-1ß (100)	1	6	nd	nd	nd
+MCP-1 (100)	46	50	nd	nd	nd
+MCP-2 (100)	28	26	nd	nd	nd
+MCP-3 (100)	58	46	nd	nd	nd
b)	**JR-FL**	**HxB2**	**MuLV**		
LW4 CD4+ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	14	68	nd		
LW5 CD4+ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	15	73	nd		

Table 1 legend:

PM1 cells were cultured as described by Lusso et al (12). Ficoll/hypaque-isolated PBMC from laboratory workers (LW) stimulated with PHA for 72h before depletion of CD8+ Lymphocytes with anti-CD8 immunomagnetic beads (DYNAL, Great Neck, NY). CD4+ Lymphocytes were maintained in culture medium containing interleukin-2 (100U/ml; Hofmann LaRoche, Nutley, NJ), as described previously (3). Target cells (1-2x10^5) were infected with supernatants (10-50ng of HIV-1 p24) from 293-cells co-transfected with an NL4/3Δenv-luciferase vector and a HIV-1 env-expressing vector (10,11). ß-Chemokines (R & D Systems, Minneapolis) were added to the target cells simultaneously with virus, at the final concentrations (ng/ml) indicated in parentheses in the first column. The ß-chemokine concentration range was selected based on prior studies (2,3). After 2h, the cells were washed twice with PBS, resuspended in ß-chemokine-containing media and maintained for 48-96h. Luciferase activity in

cell lysates was measured as described previously (10,11). The values indicated represent luciferase activity (cpm)/ng p24/mg protein, expressed relative to that in virus-control cultures lacking ß-chemokines (100%), and are the means of duplicate or sextuplicate determinations. nd, not done. R/Mα/Mß, RANTES + MIP-1α + MIP-1ß.

RANTES and MIP-1ß were strongly active when added individually, while other ß-chemokines - MlP-1α, MCP-1, MCP-2 and MCP-3 (refs. 13-15) - were weaker inhibitors (Table 1a). However, MIP-1α, MIP-1ß and RANTES, in combination, did not inhibit infection of PM1 cells by the TCLA strains NL4/3 and HxB2, or by the amphotropic murine leukemia virus (MuLV-Ampho) pseudotype (Table 1a). Thus, phenotypic characteristics of the HIV-1 envelope glycoproteins influence their sensitivity to ß-chemokines in a virus entry assay.

The *env*-complementation assay was used to assess HIV-1 entry into CD4+ T-cells from two control individuals (LW4 and LW5). MIP-1α, MIP-1ß and RANTES strongly inhibited infection by the NSI primary strain JR-FL infection of LW4's and LW5's $CD4^+$ T-cells, and weakly reduced HxB2 infection of LW cells (Table 1b), suggesting that there may be some overlap in receptor usage on activated $CD4^+$ T-cells by different virus strains. BaL env-mediated replication in normal PBL was also inhibited by MIP-1α, MIP-1ß and RANTES, albeit with significant inter-donor variation in sensitivity (data not shown).

It was determined when ß-chemokines inhibited HIV-1 replication by showing that complete inhibition of infection of PM1 cells required the continuous presence of ß-chemokines for up to 5h after addition of ADA or BaL env-complemented virus (Fig.3a). Pre-treatment of the cells with ß-chemokines for 2h or 24h prior to infection had no inhibitory effect if the cells were subsequently

washed before virus addition. Furthermore, adding ß-chemokines 2h after virus only minimally affected virus entry (Fig.3a). A PCR-based assay was next used to detect HIV-1 early DNA reverse transcripts in PM1 cells after 10h of infection; reverse transcription of ADA, but not of NL4/3, could not be detected in the presence of MIP-1ß and RANTES (Fig.3b). Thus, inhibition by ß-chemokines requires their presence during at least one of the early stages of HIV-1 replication: virus attachment, fusion and early reverse transcription.

As described in part in the First Series of Experiments, these sites of action were discriminated, first by testing whether ß-chemokines inhibited binding of JR-FL or BRU (LAI) gp120 to soluble CD4, or of tetrameric CD4-IgG2 binding to HeLa-JR-FL cells expressing oligomeric envelope glycoproteins (17). No inhibition by any of the ß-chemokines was found in either assay, whereas the OKT4a CD4-MAb was strongly inhibitory in both (Fig. 2 and data not shown). Thus, ß-chemokines inhibit a step after CD4 binding, when conformational changes in the envelope glycoproteins lead to fusion of the viral and cellular membranes (18). Cell-cell membrane fusion is also induced by the gp120-CD4 interaction, and can be monitored directly by resonance energy transfer (RET) between fluorescent dyes incorporated into cell membranes (17). In the RET assay, OKT4a completely inhibits membrane fusion of PM1 cells with HeLa cells expressing the envelope glycoproteins of either JR-FL (HeLa-JR-FL, the same cell line referred to above as HeLa-env_{JR-FL}) or BRU (HeLa-BRU, the same cell line referred to above as HeLa-env_{LAI}), confirming the specificity of the process (17). RANTES, MIP-1ß (and to a lesser extent, MIP-1α) strongly inhibited membrane fusion of HeLa-JR-FL cells with PM1 cells, whereas fusion between PM1 cells and HeLa-BRU cells was insensitive to these ß-chemokines (Fig. 1 and Table 2a).

Table 2:Effect of ß-chemokines on HIV-1 envelope glycoprotein-mediated membrane fusion measured using the RET assay

	% Fusion	
	HeLa-JR-FL	HeLa-BRU
a) PM1 cells		
no chemokines	100	100
+R/Mα/Mß (80/400/100)	1	95
+RANTES (80)	8	100
+MIP-1α (400)	39	100
+MIP-1ß (100)	13	93
+MCP-1 (100)	99	98
+MCP-2 (100)	72	93
+MCP-3 (100)	98	99
b) LW5 CD4+ cells		
no chemokines	100	100
+R/Mα/Mß(106/533/133)	39	100
+RANTES (106)	65	95
+MIP-1α (533)	72	100
+MIP-1ß (133)	44	92
+OKT4A (3ug/ml)	0	0

Table 2 legend:

CD4+ target cells (mitogen-activated CD4+ lymphocytes or PM1 cells) were labeled with octadecyl rhodamine (Molecular Probes, Eugene, OR), and HeLa-JR-FL cells, HeLa-BRU cells (or control HeLa cells, not shown) were labeled with octadecyl fluorescein (Molecular Probes), overnight at 37°C. Equal numbers of labeled target cells and env-expressing cells were mixed in 96-well plates and ß-chemokines (or CD4 MAb OKT4a) were added at the final concentrations (ng/ml) indicated in parentheses in the first column. Fluorescence emission values were determined 4h after cell mixing (17). If cell fusion occurs, the dyes are closely associated in the conjoined membrane such that excitation of fluorescein at 450nm results in resonance energy transfer (RET) and emission by rhodamine at 590nm. Percentage fusion is defined as equal to 100 x [(Exp RET - Min RET) / (Max RET - Min RET)], where Max RET = %RET obtained when HeLa-Env and CD4- cells are mixed, Exp RET = %RET obtained when HeLa-Env and CD4+ cells are mixed in the presence of fusion-inhibitory compounds, and Min RET = %RET obtained

when HeLa cells (lacking HIV-1 envelope glycoproteins) and CD4⁺ cells are mixed. The %RET value is defined by a calculation described elsewhere(17), and each is the mean of triplicate determinations. These values were, for HeLa-JR-FL and HeLa-BRU cells respectively: PM1 cells 11.5%, 10.5%; LW5 CD4⁺ cells, 6.0%, 10.5%; R/Mα/Mß, RANTES + MIP-1α + MIP-1ß.

Similar results were obtained with primary CD4⁺ T-cells from LW5 (Table 2b), although higher concentrations of ß-chemokines were required to inhibit membrane fusion in the primary cells than in PM1 cells. Thus, the actions of the ß-chemokines are not restricted to the PM1 cell line. The RET assay demonstrates that ß-chemokines interfere with env-mediated membrane fusion.

The simplest explanation of these results is that the binding of certain ß-chemokines to their receptor(s) prevents, directly or otherwise, the fusion of HIV-1 with CD4⁺ T-cells. It has been known for a decade that HIV-1 requires a second receptor for entry into CD4⁺ cells (19-21). This function is supplied, for TCLA strains, by fusin (9). Several receptors for MIP-1α, MIP-1ß and RANTES have been identified (6,7), and ß-chemokines exhibit considerable cross-reactivity in receptor usage (4-8). However, C-C CKR-1 and, especially, C-C CKR-5 were identified as the most likely candidates, based on tissue expression patterns and their abilities to bind MIP-1α, MIP-1ß and RANTES (4,7,8,15,22). C-C CKR-1, C-C CKR-5 and LESTR are each expressed at the mRNA level in PM1 cells and primary macrophages (data not shown). These and other ß-chemokine receptors were therefore PCR-amplified, cloned and expressed.

The expression of C-C CKR-5 in HeLa-CD4 (human), COS-CD4 (simian) and 3T3-CD4 (murine) cells rendered each of them readily infectible by the primary, NSI strains ADA and BaL in the env-complementation assay of HIV-1 entry (Table 3).

Table 3: C-C CKR-5 expression permits infection of CD4-expressing cells by primary, NSI HIV-1 strains

									R/Mα/Mß
		pcDNA3.1	LESTR	CKR-1	CKR-2a	CKR-3	CKR-4	CKR-5	CKR-5
COS-CD4	ADA	798	456	600	816	516	534	153000	3210
	BaL	660	378	600	636	516	618	58800	756
	HxB2	5800	96700	5240	5070	5470	5620	4850	5000
HeLa-CD4	ADA	678	558	4500	912	558	600	310000	6336
	BaL	630	738	1800	654	516	636	104000	750
	HxB2	337000	nd	nd	nd	nd	nd	nd	356000
3T3-CD4	ADA	468	558	450	618	534	606	28400	1220
	BaL	606	738	660	738	534	558	11700	756
	HxB2	456	24800	618	672	732	606	618	606

Table 3 legend:

Chemokine receptor genes C-C CKR-1, C-C CKR-2a, C-C CKR-3, C-C CKR-4 and C-C CKR-5 have no introns (4-8,15,22) and were isolated by PCR performed directly on a human genomic DNA pool derived from the PBMC of seven healthy donors. Oligonucleotides overlapping the ATG and the stop codons and containing BamHI and XhoI restriction sites for directional cloning into the pcDNA3.1 expression vector (Invitrogen Inc.) were used. LESTR (also known as fusin or HUMSTR) (4,9,24) was cloned by PCR performed directly on cDNA derived from PM1 cells, using sequences derived from the NIH database. Listed below are the 5'and 3' primer pairs used in first (5-1 and 3-1) and second (5-2 and 3-2) round PCR amplification of the CKR genes directly from human genomic DNA, and of LESTR from PM1 cDNA. Only a single set of primers was used to amplify CKR-5.

LESTR: L/5-1 = AAG CTT GGA GAA CCA GCG GTT ACC ATG GAG GGG ATC (SEQ ID NO: 6);

L/5-2 = GTC TGA GTC TGA GTC AAG CTT GGA GAA CCA (SEQ ID NO: 7);

L/3-1 = CTC GAG CAT CTG TGT TAG CTG GAG TGA AAA CTT GAA GAC TC (SEQ ID NO: 8);

L/3-2 = GTC TGA GTC TGA GTC CTC GAG CAT CTG TGT (SEQ ID NO: 9);

CKR-1:C1/5-1 = AAG CTT CAG AGA GAA GCC GGG ATG GAA ACT CC (SEQ ID NO: 10);

C1/5-2 = GTC TGA GTC TGA GTC AAG CTT CAG AGA GAA (SEQ ID NO: 11);

C1/3-1 = CTC GAG CTG AGT CAG AAC CCA GCA GAG AGT TC (SEQ ID NO: 12);

C1/3-2 = GTC TGA GTC TGA GTC CTC GAG CTG AGT CAG (SEQ ID NO: 13);

CKR-2a:C2/5-1 = AAG CTT CAG TAC ATC CAC AAC ATG CTG TCC AC (SEQ ID NO: 14);

C2/5-2= GTC TGA GTC TGA GTC AAG CTT CAG TAC ATC (SEQ ID NO: 15);

C2/3-1 = CTC GAG CCT CGT TTT ATA AAC CAG CCG AGA C (SEQ

ID NO: 16);
C2/3-2 = GTC TGA GTC TGA GTC CTC GAG CCT CGT TTT (SEQ ID NO: 17);
CKR-3: C3/5-1 = AAG CTT CAG GGA GAA GTG AAA TGA CAA CC (SEQ ID NO: 18);
C3/5-2= GTC TGA GTC TGA GTC AAG CTT CAG GGA GAA (SEQ ID NO: 19);
C3/3-1 = CTC GAG CAG ACC TAA AAC ACA ATA GAG AGT TCC (SEQ ID NO: 20);
C3/3-2 = GTC TGA GTC TGA GTC CTC GAG CAG ACC TAA (SEQ ID NO: 21);
CKR-4: C4/5-1 = AAG CTT CTG TAG AGT TAA AAA ATG AAC CCC ACG G (SEQ ID NO: 22);
C4/5-2 = GTC TGA GTC TGA GTC AAG CTT CTG TAG AGT (SEQ ID NO: 23);
C4/3-1 = CTC GAG CCA TTT CAT TTT TCT ACA GGA CAG CAT C (SEQ ID NO: 24);
C4/3-2 = GTC TGA GTC TGA GTC CTC GAG CCA TTT CAT (SEQ ID NO: 25);
CKR-5: C5/5-12 = GTC TGA GTC TGA GTC AAG CTT AAC AAG ATG GAT TAT CAA (SEQ ID NO: 26);
C5/3-12 = GTC TGA GTC TGA GTC CTC GAG TCC GTG TCA CAA GCC CAC (SEQ ID NO: 37).

The human CD4-expressing cell lines HeLa-CD4 (P42), 3T3-CD4 (sc6) and COS-CD4 (Z28T1) (23) were transfected with the different pcDNA3.1-CKR constructs by the calcium phosphate method, then infected 48h later with different reporter viruses (200ng of HIV-1 p24/10^6 cells) in the presence or absence of ß-chemokines (400ng/ml each of RANTES, MIP-1α and MIP-1ß). Luciferase activity in cell lysates was measured 48h later (10,11). ß-Chemokine blocking data is only shown for C-C CKR-5, as infection mediated by the other C-C CKR genes was too weak for inhibition to be quantifiable. In PCR-based assays of HIV-1 entry, a low level of entry of NL4/3 and ADA into C-C CKR-1 expressing cells (data not shown) was consistently observed.

Neither LESTR nor C-C CKR-1, -2a, -3 or -4 could

substitute for C-C CKR-5 in this assay. The expression of LESTR in COS-CD4 and 3T3-CD4 cells permitted HxB2 entry, and HxB2 readily entered untransfected (or control plasmid-transfected) HeLa-CD4 cells (Table 3). Entry of BAL and ADA into all three C-C CKR-5-expressing cell lines was almost completely inhibited by the combination of MIP-1α, MIP-1ß and RANTES, whereas HxB2 entry into LESTR-expressing cells was insensitive to ß chemokines (Table 3). These results suggest that C-C CKR-5 functions as a ß-chemokine-sensitive second receptor for primary, NSI HIV-1 strains.

The second receptor function of C-C CKR-5 was confirmed in assays of env-mediated membrane fusion. When C-C CKR-5 was transiently expressed in COS and HeLa cell lines that permanently expressed human CD4, both cell lines fused strongly with HeLa cells expressing the JR-FL envelope glycoproteins, whereas no fusion occurred when control plasmids were used (data not shown). Expression of LESTR instead of C-C CKR-5 did not permit either COS-CD4 or HeLa-CD4 cells to fuse with HeLa-JR-FL cells, but did allow fusion between COS-CD4 cells and HeLa-BRU cells (data not shown).

The fusion capacity of ß-chemokine receptors was also tested in the RET assay. The expression of C-C CKR-5, but not of C-C CKR-1, -2a, -3 or -4, permitted strong fusion between HeLa-CD4 cells and HeLa-JR-FL cells. The extent of fusion between HeLa-JR-FL cells and C-C CKR-5-expressing HeLa-CD4 cells was greater than the constitutive level of fusion between HeLa-BRU cells and HeLa-CD4 cells (Fig.4). The fusion-conferring function of C-C CKR-5 for primary, NSI HIV-1 strains has therefore been confirmed in two independent fusion assays.

Experimental Discussion

Together, the above results establish that MIP-1α, MIP-1ß and RANTES inhibit HIV-1 infection at the entry stage, by interfering with the virus-cell fusion reaction subsequent to CD4 binding. It was also shown that C-C CKR-5 can serve as a second receptor for entry of primary NSI strains of HIV-1 into CD4+ T-cells, and that the interaction of ß-chemokines with C-C CKR-5 inhibits the HIV-1 fusion reaction.

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THIRD SERIES OF EXPERIMENTS

The chemokine SDF-1 (stromal cell-derived factor 1) is the natural ligand for Fusin/CXCR4 and blocks infection by laboratory-adapted strains of HIV-1 (Ref. 1 and 2). SDF-1 exists as at least two forms, SDF-1α and SDF-1ß based on variable splicing of the SDF-1 gene (Ref. 1 and 3) In the RET assay, this chemokine specifically inhibits membrane fusion mediated by gp120/gp41 form the laboratory-adapted strain HIV_{LAI} but not by gp120/gp41 from the macrophage-tropic isolate HIV-1_{JR-FL} as shown in Figure 5.

References of the Third Series of Experiments

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FOURTH SERIES OF EXPERIMENTS

Direct Binding of HIV-1_{JR-FL} gp120 to $CCR5^+$ $CD4^-$ Cells

The direct binding of HIV-1_{JR-FL} gp120 to $CCR5^+$ $CD4^-$ cells has been demonstrated. In this case, preincubation of the gp120 with sCD4 or another CD4-based molecule is required, presumably because this results in a conformational change in gp120 that exposes a chemokine receptor binding site. Figure 6 illustrates the use of flow cytometry to measure the direct binding of sCD4/gp120 complexes to human CCR5-bearing murine L1.2 cells. Background levels of binding were observed with either biotinylated protein alone, or if gp120 from the laboratory-adapted strain HIV-1_{LAI} is used in place of the HIV-1_{JR-FL} gp120 (data not shown).

This assay has been adapted for drug screening purposes to a 96-well microplate format where binding of the sCD4/gp120 complexes to $CCR5^+/CD4^-$ cells is measured using a fluorometric plate reader. One method is as follows:

1) Plate out L1.2-$CCR5^+$ cells (approx. 500,000/well).
2) Add inhibitor for 1 hour at room temperature.
3) Wash and add biotinylated sCD4(2.5μg/ml) and biotinylated HIV-1_{JR-FL} gp120 (5μg/ml), then incubate for 2 hours at room temperature.
4) Wash and incubate with streptavidin-phycoerythrin (100ng/nl).
5) Wash and measure the amount of bound gp120/sCD4 using a fluorometric plate reader exciting at 530nm and reading emission at 590nm.

Using this method, inhibition of binding of gp120/sCD4 to CCR5 by CC-chemokines (Fig. 7) and antibodies to CCR5 that block HIV-1 infection (not shown) have been demonstrated.

Inhibition of HIV-1 envelope-mediated membrane fusion by the bicyclam, JM3100.

The bicyclam JM3100, obtained from Dr. J. Moore (Aaron Diamond AIDS Research Center, NY) was tested for ability to inhibit membrane fusion mediated by the envelope glycoproteins of the LAI or JR-FL strains of HIV-1 using the resonance energy transfer (RET) assay described above. As illustrated in Fig. 7, this molecule specifically and potently inhibits fusion mediated by gp120/gp41 from the HIV-1_{LAI} strain, and not from the HIV-1_{JR-FL} strain. These data suggest that this molecule specifically inhibits HIV fusion by blocking the interaction between HIV-1_{LAI} gp120 and CXCR4.

FIFTH SERIES OF EXPERIMENTS

CCR5 Receptor Binding Assay

Materials:

1. $CCR5^+$/L1.2 cell line
2. L1.2 cell line
3. JRFL-gp120, biotinylated
4. sCD4, unconjugated (Intracell, Cat #13101)
5. 96-well round bottom plate (Corning, cat #25850)
6. Streptavidin, phycoerythrin conjugated [SA-PE] (Becton Dickinson, cat #349023)
7. PBS without Calcium and Magnesium [PBS(-)] (Gibco BRL, cat #14190)

Method:

1. Culture $CCR5^+$ and parental L1.2 cells and treat with sodium butyrate as described (Wu et al., J. Exp. Med 185:1681).

2. Add cells to 96-well plate (~$3x10^5$ cells/well)

3. Centrifuge plate and remove supernatant.

4. Dilute inhibitory compounds as desired in PBS(-)/0.1% NaN_3. Add 40 μl of inhibitory compounds to cells. Add 40 μl of PBS(-)/0.1% NaN_3 to wells without inhibitory compounds.

5. Shake plate to suspend cells in solution. Incubate at room temperature for 1 hour.

6. Prepare an equimolar (~50nM) mixture of sCD4 and biotinylated gp120. Add 40μl of sCD4:biotinylated gp120 complex per well. (Final volume in well = 80μl). Shake plate to suspend cells in protein solution. Incubate at room temperature for one hour.

7. Centrifuge plate and remove supernatant. Add 200μl of PBS(-)0.1% NaN_3 per well. Repeat this washing procedure, for a total of three washes.

8. Centrifuge plate and remove supernatant. Dilute SA-PE 1:50 in PBS(-)/0.1% NaN_3 and add 40μl of diluted reagent to cells. Shake plate to suspend cells in solution. Incubate at room temperature for one hour.

9. Centrifuge plate as above and remove supernatant. Add 200μl of PBS(-)/0.1% NaN_3 per well. Repeat this washing procedure for a total of three washes.

10. Centrifuge plate as above and remove supernatant. Add 200μl of PBS(-)/0.1% NaN_3 per well.

11. Centrifuge plate and measure the fluorescence. Emission at 590nm following excitation at 530nm.

12. % Inhibition is calculated by using the following formula:

%Inhibition = [Max -Reading] /[Max-Min]

Max = Average of values in wells containing [sCD4: biotinylated gp120 w/CCR5+/L1.2 cells, no inhibitor]

Min = Average of values in wells containing sCD4:biotinylated gp120 w/ L1.2 cells, no inhibitor.

Reading = Value in specific well

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Progenics Pharmaceuticals, Inc.

(ii) TITLE OF INVENTION: A Method For Preventing HIV-1 Infection of CD4+ Cells

(iii) NUMBER OF SEQUENCES: 27

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 1185 Avenue of the Americas
(C) CITY: New York
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(E) COUNTRY: USA
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: White, John P
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(C) REFERENCE/DOCKET NUMBER: 50875-F-PCT/JPW/AKC

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAGGCTACT TCCCTGATTG GCAGAACTAC ACACCAGG 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCAAGCCGA GTCCTGCGTC GAGAG 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGACTTTCC GCTGGGGACT TTC
23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCTGTTCGGG CGCCACTGCT AGAGATTTTC CAC 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: n/a
(D) TOPOLOGY: n/a

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
1 5 10 15

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe Val
20 25 30

Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg
35 40 45 50

Glu Tyr Ile Asn Ser Leu Glu Met Ser
55 60

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGCTTGGAG AACCAGCGGT TACCATGGAG GGGATC 36

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCTGAGTCT GAGTCAAGCT TGGAGAACCA 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCGAGCATC TGTGTTAGCT GGAGTGAAAA CTTGAAGACT C 41

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTCTGAGTCT GAGTCCTCGA GCATCTGTGT 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTTCAGA GAGAAGCCGG GATGGAAACT CC 32

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTCTGAGTCT GAGTCAAGCT TCAGAGAGAA 30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCGAGCTGA GTCAGAACCC AGCAGAGAGT TC 32

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCTGAGTCT GAGTCCTCGA GCTGAGTCAG 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGCTTCAGT ACATCCACAA CATGCTGTCC AC 32

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCTGAGTCT GAGTCAAGCT TCAGTACATC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCGAGCCTC GTTTTATAAA CCAGCCGAGA C 31

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCTGAGTCT GAGTCCTCGA GCCTCGTTTT 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGCTTCAGG GAGAAGTGAA ATGACAACC 29

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTCTGAGTCT GAGTCAAGCT TCAGGGAGAA 30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTCGAGCAGA CCTAAAACAC AATAGAGAGT TCC 33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTCTGAGTCT GAGTCCTCGA GCAGACCTAA 30

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AAGCTTCTGT AGAGTTAAAA AATGAACCCC ACGG 34

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCTGAGTCT GAGTCAAGCT TCTGTAGAGT 30

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCGAGCCAT TTCATTTTTC TACAGGACAG CATC 34

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTCTGAGTCT GAGTCCTCGA GCCATTTCAT 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCTGAGTCT GAGTCAAGCT TAACAAGATG GATTATCAA 39

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotides

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTCTGAGTCT GAGTCCTCGA GTCCGTGTCA CAAGCCCAC 39

What is claimed is:

1. A non-chemokine agent capable of binding to a chemokine receptor and inhibiting fusion of HIV-1 to $CD4^+$ cells with the proviso that the agent is not a known bicyclam or its known derivative.

2. The non-chemokine agent of claim 1, wherein the non-chemokine agent is a oligopeptide.

3. The non-chemokine agent of claim 1, wherein the non-chemokine agent is a nonpeptidyl agent.

4. The non-chemokine agent of claim 1, wherein the non-chemokine agent is a polypeptide.

5. The non-chemokine agent of claim 4, wherein the polypeptide is an antibody or a portion of an antibody.

6. The non-chemokine agent of claim 4, wherein the polypeptide comprises amino acid sequence as set forth in SEQ ID NO:5.

7. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first seven N-terminal amino acids of said sequence.

8. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first eight N-terminal amino acids of said sequence.

9. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first nine N-terminal amino acids of said sequence.

10. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the deletion of the first ten N-terminal amino acids of said sequence.

11. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide.

12. The non-chemokine agent of claim 4, wherein the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by removing the N-terminal alanine and replacing it by serine or threonine and an additional amino acid or oligopeptide or nonpeptidyl moiety.

13. The non-chemokine agent of claim 11 or 12, wherein the additional amino acid is methionine.

14. An non-chemokine agent capable of binding to CXCR4 and inhibiting HIV-1 infection with the proviso that the agent is not a known bicyclam or its known derivative.

15. The non-chemokine agent of claim 14, wherein the agent is an oligopeptide.

16. The non-chemokine agent of claim 14, wherein the agent is a polypeptide.

17. The non-chemokine agent of claim 16, wherein the polypeptide comprises the SDF-1 sequence with the deletion of the first six N-terminal amino acids of said sequence.

18. The non-chemokine agent of claim 16, wherein the polypeptide comprises the SDF-1 sequence with the

deletion of the first seven N-terminal amino acids of said sequence.

19. The non-chemokine agent of claim 16, wherein the polypeptide comprises the SDF-1 sequence with the deletion of the first eight N-terminal amino acids of said sequence.

20. The non-chemokine agent of claim 16, wherein the polypeptide comprises the SDF-1 sequence with the deletion of the first nine N-terminal amino acids of said sequence.

21. The non-chemokine agent of claim 16, wherein the N-terminal glycine of SDF-1 is replaced by serine and derivatized with biotin.

22. The non-chemokine agent of claim 16, wherein the N-terminal glycine of SDF-1 is replaced by serine and derivatized with methionine.

23. The non-chemokine agent of claim 16, wherein the N-terminus of SDF-1 is modified by the addition of a methionine before the terminal glycine.

24. The agent of claim 16, wherein the agent is an antibody or a portion of an antibody.

25. The agent of claim 14, wherein the agent is a non-peptidyl agent.

26. A pharmaceutical composition comprising an amount of the non-chemokine agent of claim 1 effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising an amount of the non-chemokine agent of claim 14 effective to

inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

28. A composition of matter capable of binding to a chemokine receptor and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the $CD4^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not inhibit the binding of the ligand to the other receptor.

29. The composition of matter of claim 28, wherein the cell surface receptor is CD4.

30. The composition of matter of claim 28, wherein the ligand comprises an antibody or a portion of an antibody.

31. A pharmaceutical composition comprising an amount of the composition of matter of claim 28 effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

32. A method for reducing the likelihood of HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 26, 27, or 31 to the subject.

33. A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 26 or 27 to the subject.

34. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) contacting an appropriate concentration of an agent with a chemokine receptor or a portion

thereof under conditions permitting the binding of the agent to the chemokine receptor;

(b) contacting the chemokine receptor resulting from step (a) with a gp120/CD4 complex under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(c) measuring the amount of bound gp120/CD4 complex wherein a decrease in the amount compared with the amount determined in the absence of the agent indicates that the agent is capable of inhibiting HIV-1 infection.

35. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) fixing a chemokine receptor on a solid matrix;

(b) contacting the agent with the fixed chemokine receptor under conditions permitting the binding of the agent to the chemokine receptor;

(c) removing the unbound agent;

(d) contacting the fixed chemokine receptor resulting in step (c) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(e) measuring the amount of bound gp120/CD4 complex; and

(f) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating

that the agent is capable of inhibiting HIV-1 infection.

36. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) fixing a chemokine receptor on a solid matrix;

(b) contacting the agent with the fixed chemokine receptor;

(c) contacting the mixture in step (b) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(d) measuring the amount of bound gp120/CD4 complex; and

(e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

37. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) contacting the agent with a gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex;

(b) contacting the gp120/CD4 complex resulting from step (a) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(c) measuring the amount of bound chemokine receptor, wherein a decrease of the amount when compared with the amount determined in the absence of the agent indicates that the agent is capable of inhibiting HIV-1 infection.

38. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) fixing a gp120/CD4 complex on a solid matrix

(b) contacting the agent with the fixed gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex;

(c) removing unbound agent;

(d) contacting the fixed gp120/CD4 complex resulting from step (c) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(e) measuring the amount of bound chemokine receptor; and

(f) comparing the amount determined in step (e) with thte amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

39. A method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of:

(a) fixing a gp120/CD4 complex on a solid matrix;

(b) contacting the agent with the fixed gp120/CD4 complex;

(c) contacting the mixture in step (b) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent;

(d) measuring the amount of bound chemokine receptor; and

(e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

40. The method of claim 34, 35, 36, 37, 38 or 39 wherein the CD4 is a soluble CD4.

41. The method of claim 34, 35, 36, 37, 38 or 39 wherein the chemokine receptor is CCR5.

42. The method of claim 34, 35, 36, 37, 38 or 39 wherein the chemokine receptor is CXCR4.

43. The method of claim 34, 35, 36, 37, 38 or 39 wherein the chemokine receptor is expressed on a cell.

44. The method of claim 43, wherein the chemokine receptor is embedded in liposomes.

45. The method of claim 43, wherein the chemokine receptor is embedded in a membrane derived from cells expressing the chemokine receptor.

46. The method of claim 43, wherein the cell is a L1.2 cell.

47. The method of claim 35 or 36, wherein the gp120, CD4 or both are labelled with a detectable marker.

48. The method of claim 37, 38 or 39, wherein the chemokine receptor is labelled with a detectable marker.

49. The method of claim 47 or 48, wherein the gp120, CD4 or the chemokine receptor is labelled with biotin.

50. The method of claim 49, wherein the biotinylated gp120, CD4 or the chemokine receptor is detected by:

(i) incubating with streptavidin-phycoerythrin,

(ii) washing the incubated mixture resulting from step (i), and

(iii) measuring the amount of bound gp120, CD4 or the chemokine receptor using a fluorometer, exciting at 530nm and reading the emission at 590nm.

51. The agent determined to be capable of inhibiting HIV-1 infection by the method of claim 34, 35, 36, 37, 38 or 39 which is previously unknown.

52. A pharmaceutical composition comprising the agent determined to be capable of inhibiting HIV-1 infection by the method of claim 34, 35, 36, 37, 38 or 39 and a pharmaceutically acceptable carrier.

53. The method of claim 34, 35, 36, 37, 38 or 39 wherein the agent is an oligopeptide.

54. The method of claim 34, 35, 36, 37, 38 or 39 wherein the agent is a polypeptide.

55. The method of claim 34, 35, 36, 37, 38 or 39 wherein the agent is a nonpeptidyl agent.

56. The agent of claim 51 linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent.

57. The agent of claim 56, wherein the compound is polyethylene glycol.

58. A pharmaceutical composition comprising an amount of the agent of claim 56 effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

59. A method for reducing the likelihood of HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 52 or 58 to the subject.

60. A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 52 or 58 to the subject.

METHOD FOR PREVENTING HIV-1 INFECTION OF CD4$^+$ CELLS

Abstract of the Disclosure

This invention provides methods for inhibiting fusion of HIV-1 to CD4$^+$ cells which comprise contacting CD4$^+$ cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4$^+$ cells is inhibited. This invention also provides methods for inhibiting HIV-1 infection of CD4$^+$ cells which comprise contacting CD4$^+$ cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the CD4$^+$ cells is inhibited, thereby inhibiting the HIV-1 infection. This invention provides non-chemokine agents capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4$^+$ cells. This invention also provides pharmaceutical compositions comprising an amount of the non-chemokine agent capable of binding to the chemokine receptor and inhibiting fusion of HIV-1 to CD4$^+$ cells effective to prevent fusion of HIV-1 to CD4$^+$ cells and a pharmaceutically acceptable carrier.

FIG. 1A

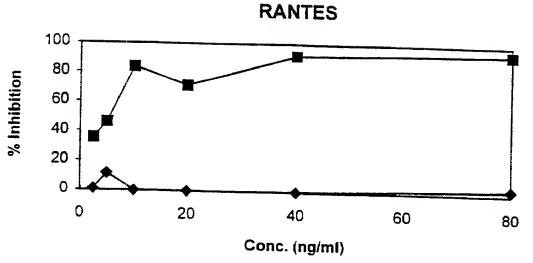


FIG. 1B

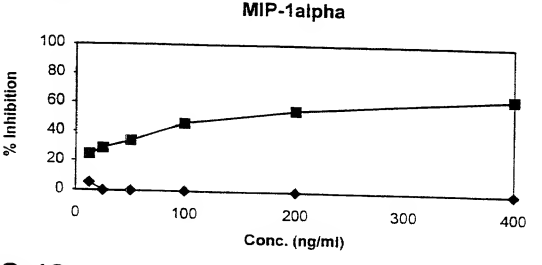


FIG. 1C

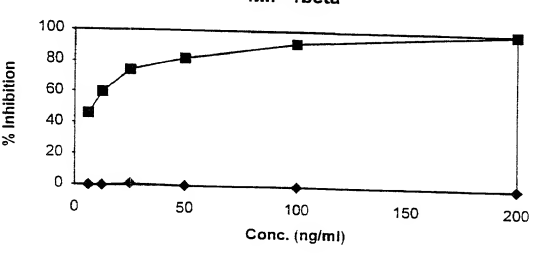


FIG. 2A

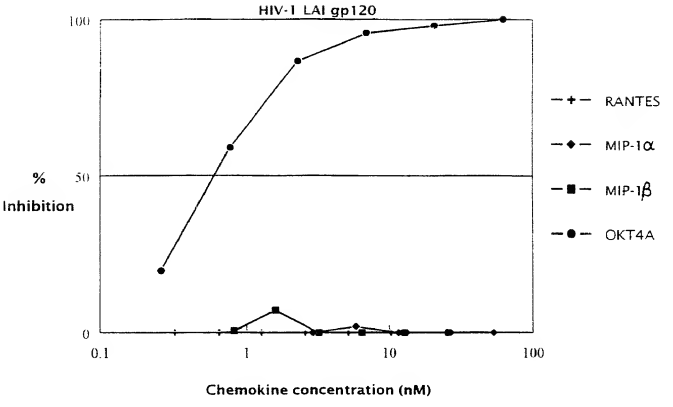

HIV-1 LAI gp120
100
50
0
% Inhibition
0.1
1
10
100
Chemokine concentration (nM)
RANTES
MIP-1α
MIP-1β
OKT4A

FIG. 2B

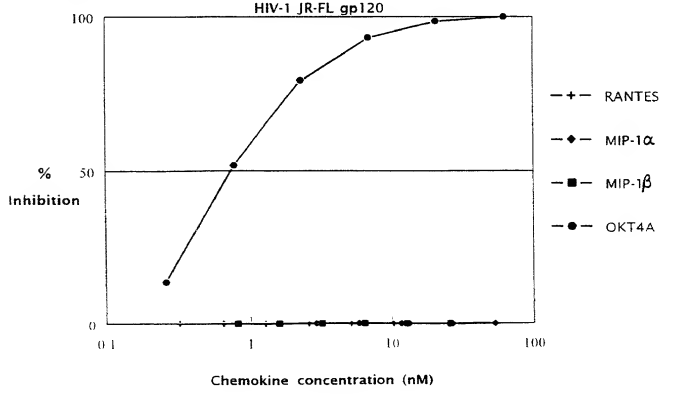
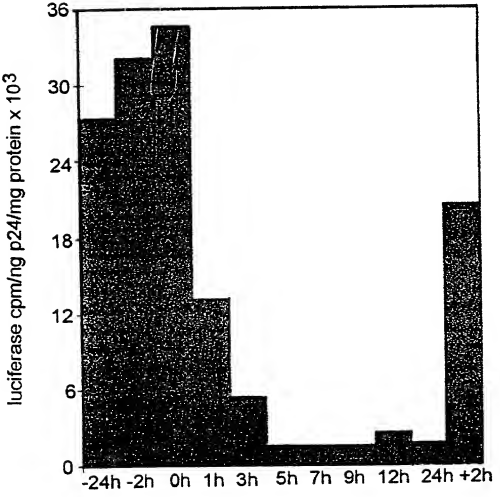

HIV-1 JR-FL gp120
100
50
0
% Inhibition
0.1
1
10
100
Chemokine concentration (nM)
RANTES
MIP-1α
MIP-1β
OKT4A

FIG. 3A


luciferase cpm/ng p24/mg protein x 10³
36
30
24
18
12
6
0
-24h -2h 0h 1h 3h 5h 7h 9h 12h 24h +2h

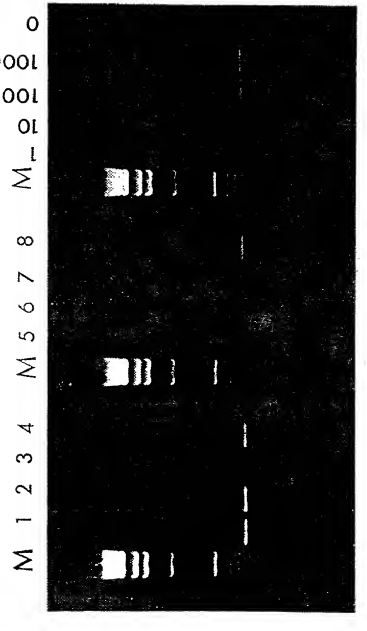


FIG. 3B

FIG. 4

FIG. 5

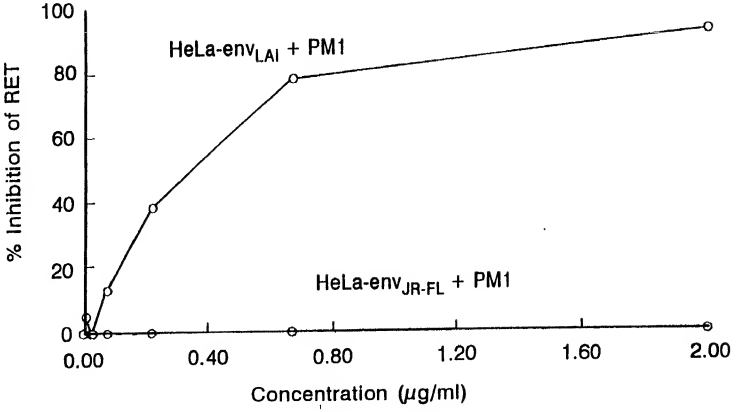


FIG. 6A

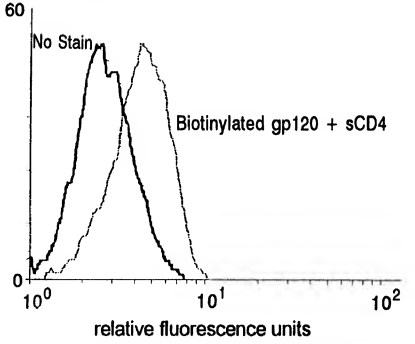

60
No Stain
Biotinylated gp120 + sCD4
0
10^0
10^1
10^2
relative fluorescence units

FIG. 6B

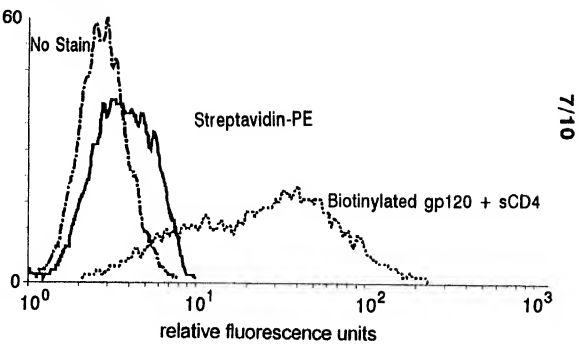

60
No Stain
Streptavidin-PE
Biotinylated gp120 + sCD4
0
10^0
10^1
10^2
10^3
relative fluorescence units

FIG. 7

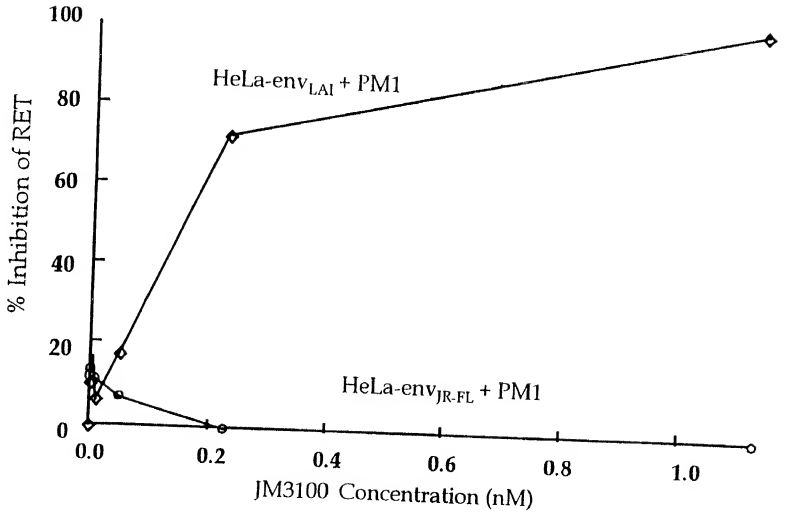

100
80
60
40
20
0
% Inhibition of RET
HeLa-env$_{LAI}$ + PM1
HeLa-env$_{JR-FL}$ + PM1
0.0
0.2
0.4
0.6
0.8
1.0
JM3100 Concentration (nM)

FIG. 8

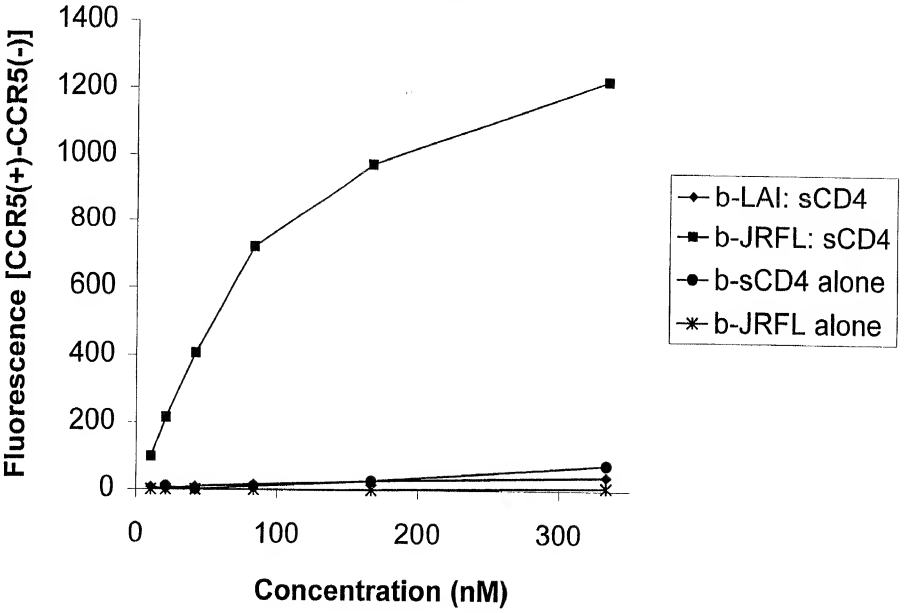
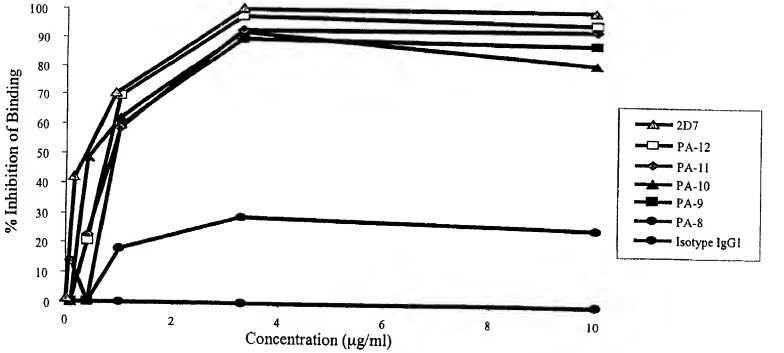

Fluorescence [CCR5(+)-CCR5(-)]
1400
1200
1000
800
600
400
200
0
0
100
200
300
Concentration (nM)
b-LAI: sCD4
b-JRFL: sCD4
b-sCD4 alone
b-JRFL alone

FIG. 9

Anti-CCR5 mAbs Inhibit gp120/CCR5 Binding



DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that

My residence, post office address, and citizenship are as stated below next to my name

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR PREVENTING HIV-1 INFECTION OF CD4+ CELLS

the specification of which:
(check one)

X *is attached hereto*

___ *was filed on* _____ *as*

Application Serial No _____

and was amended _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37 Code of Federal Regulations, Section 1.56

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed:

Prior Foreign Application(s)			*Priority Claimed*	
Number	*Country*	*Filing Date*	*Yes*	*No*
PCT/US98/12331		June 12, 1998	X	

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

Provisional Application No.	*Filing Date*	*Status*
60/019,715	June 14, 1996	Abandoned as of June 15, 1997
60/014,532	April 2, 1996	Abandoned as of April 3, 1997

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:

Application Serial No.	*Filing Date*	*Status*
PCT/US98 12331	June 12, 1998	Pending as of June 12, 1998
US 08/876,078	June 13, 1997	Pending as of June 13, 1997
US 08/831,823	April 2, 1997	Pending as of April 2, 1997

And I hereby appoint

John P. White (Reg. No. 28,678); Christopher C. Dunham (Reg. No. 22,031); Norman H. Zivin (Reg. No. 25,385); Jay H. Maioli (Reg. No. 27, 213); William E. Pelton (Reg. No. 25, 702); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No. 33, 970); Robert T. Maldonado (Reg. No. 38,232); Paul Teng (40.837); Gary J. Gershik (Reg. No. 39,992); Richard F. Jaworski (Reg. No. 33, 515); Elizabeth M. Wiechowski (Reg. No., 42, 226); and Pedro C. Fernandez (Reg. No. 41, 741)

and each of them, all c/o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

John P. White *Reg No.* 28,678

Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
Tel. (212) 278-0400

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first joint inventor Graham P. Allaway

Inventor's signature _____

Citizenship Great Britain *Date of signature* _____

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Post Office Address same as above

Full name of joint inventor (if any) Virginia M. Litwin

Inventor's signature _____

Citizenship U.S.A. *Date of signature* _____

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Inventor's signature _____

Citizenship U.S.A. *Date of signature* _____

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Post Office Address same as above

Full name of joint inventor (if any) William C. Olson

Inventor's signature _____

Citizenship U.S.A. *Date of signature* _____

Residence 21 Fawn Court, Ossining, New York 10562

Post Office Address same as above

_____

Full name of joint inventor (if any) _____

Inventor's signature _____

Citizenship _____ *Date of signature* _____

Residence _____

Post Office Address _____

_____

Full name of joint inventor (if any) _____

Inventor's signature _____

Citizenship _____ *Date of signature* _____

Residence _____

Post Office Address _____

_____